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CROSSLINKING ALBUMIN FOR DRUG RELEASE FROM SPRAY DRIED PARTICLES

By

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CROSSLINKING ALBUMIN FOR DRUG RELEASE FROM SPRAY DRIED PARTICLES

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ABSTRACT

In space, astronauts are exposed to a large doses of ionizing radiation which can cause various health problems. The drug delivery of antioxidants and anti-inflammatory substances is a promising countermeasure for the harmful cellular effects of radiation exposure. Curcumin is a polyphenol derived from the rhizome of the turmeric plant that has strong antioxidant capabilities. The therapeutic potential of this radical scavenging drug is limited by poor uptake in the body due to its insolubility in water, rapid metabolism by the intestinal mucosa and liver, and quick excretion. Drug delivery vehicles can be used to enhance the bioavailability of curcumin. Albumin, a biodegradable and non-immunogenic plasma protein, can be utilized as a drug delivery vehicle. Curcumin binds to albumin's hydrophobic pockets, which increases its solubility and decreases its rate of degradation in physiological conditions. Curcumin was solubilized with 0.5% (w/v) fatty acid free human serum albumin (FAF HSA) and spray dried to form a dry powder of particles. To produce particles with a smooth and spherical morphology, 0.05% (v/v) Tween® 20 was included in the solution. Curcumin release from these particles followed a first-order release profile ($C_t/C_{inf} = 1 - e^{kt}$, t = time in min) with $k = 0.065 \pm 0.003$.

To alter the release of curcumin from the spray dried particles, 0.5% FAF HSA was crosslinked using 0.01M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 5 mM N-hydroxysulfosuccinimide. This crosslinking method was confirmed by native polyacrylamide gel electrophoresis (PAGE) and differential scanning calorimetry (DSC). For the native PAGE results, multiple protein bands at high molecular weights were observed for the crosslinked FAF HSA and a single protein band for the uncrosslinked

FAF HSA. This suggested that multiple FAF HSA molecules had been crosslinked to each other. DSC results reported a significant increase in melting point from 53.60 ± 1.35 °C to 63.82 ± 2.34 °C of spray dried FAF HSA particles, further confirming the crosslinking method. Curcumin was bound to the crosslinked FAF HSA in the presence of 0.05% Tween® 20 and spray dried. The resulting particles showed a less uniform morphology and curcumin release followed a first-order profile with a significantly lower $k = 0.049 \pm 0.004$. Future work to improve the morphology of the crosslinked FAF HSA particles and increase the level of crosslinking to further slow curcumin release will enhance the applicability of these particles in mitigating radiation-induced cell damage.

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NOMENCLATURE

BHT	= 2,6-Di-*tert*-butyl-4-methylphenol
BS^3	= bis(sulfosuccinimidyl) suberate
BSA	= bovine serum albumin
CEW	= chicken egg white
DI	= deionized
DLS	= dynamic light scattering
DMSO	= dimethyl sulfoxide
DSC	= differential scanning calorimetry
EDC	= 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide
EHT	= extra-high tension
FAF	= fatty acid free
HSA	= human serum albumin
MWCO	= molecular weight cutoff
PAGE	= polyacrylamide gel electrophoresis
PBS	= phosphate buffered saline
SEM	= standard error of the mean
SSE	= sum of squared errors of prediction
sulfo-NHS	= *N*-hydroxysulfosuccinimide
T20	= Tween® 20, for electrophoresis
UV-Vis	= ultraviolet-visible
λ	= wavelength

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I. INTRODUCTION

A. Background

Astronauts are exposed to increased levels of ionizing radiation while in space. With this increased exposure comes a multitude of health risks including the potential to develop cancer (Durante and Cucinotta, 2008). TABLE I outlines approximations for radiation doses experienced during space flight as compared to radiation doses experienced on Earth during the same period of time (Cucinotta and Durante, 2006; Rask, 2008). As can be seen, protection from radiation becomes increasingly important as missions become longer and take humans deeper into space. The development of effective countermeasures is critical to advancing human deep space exploration.

There are three main methods for limiting exposure to ionizing radiation. The first is to move further away from the radiation source. This is not possible in space because space radiation is omnidirectional. The second way to reduce exposure is to decrease the exposure time, but manned missions will only get longer as humans travel farther away from Earth to other planets, asteroids, and beyond. The last method for limiting exposure to ionizing radiation is shielding. Though plausible, the additional mass from the shielding would likely exceed current launch capabilities. Attempts are made to minimize harm via strategic launch schedules and astronaut selection of individuals with lower susceptibility to the effects of radiation exposure (Cucinotta and Durante, 2009).

TABLE I

OBSERVED RADIATION DOSE FOR VARIOUS SPACE TRAVEL MISSIONS

Mission Type	**Mission Radiation Dose**	**Terrestrial Radiation Dose (3.6 mSv/year)**
Apollo 14 (9-day mission to the Moon)	11.4 mSv	0.09 mSv
International Space Station Mission (up to 6 months orbiting Earth at 353 km)	160 mSv	1.8 mSv
Estimated Mars Mission (3 years)	1,200 mSv	10.8 mSv

Radiation can cause damage to cellular components through direct energy transfer or indirect production of free radicals from water molecules (Cucinotta and Durante, 2009). Cellular damage is followed by an inflammatory response. Most chronic diseases are the result of chronic dysregulation of the inflammatory response (Aggarwal, 2007). Since exposure to radiation during space travel is currently unavoidable, countermeasures that work to oppose these harmful effects become increasingly important. The development of delivery systems for antioxidant and anti-inflammatory drugs is one such countermeasure.

Curcumin (diferuloylmethane) is a highly active polyphenol component of *Curcuma longa* (commonly known as turmeric), an herb frequently used as a food additive and preservative. It comes from the dried rhizome of the turmeric plant, and demonstrates chemopreventative activities as a result of its antioxidant and anti-inflammatory properties. Curcumin is a good scavenger of free radicals. Additionally, it can inhibit expression or suppress production of pro-inflammatory markers (Anand et al., 2007). It

does not show toxicity and has been safely tested at dosage levels of 8 g/day in humans (Cheng et al., 2001).

The therapeutic potential of curcumin is limited by its poor bioavailability. These bioavailability limitations can be attributed to curcumin's virtual insolubility in biological fluids and rapid degradation at physiologic pH (Hatcher et al., 2008; Sharma et al., 2005). The oral intake of 3.6 g of curcumin, has been shown to peak serum curcumin levels at 11.1 nmol/L after one hour of dosing (Sharma et al., 2004). At physiologic pH, curcumin degradation into feruloylmethane and vanillin occurs within 30 minutes (Lin et al., 2000).

Intravenous administration of curcumin would be a preferred route of delivery because it would allow curcumin to bypass rapid degradation by the intestinal mucosa (Anand et al., 2007). For this administration to work, curcumin's solubility in biological fluids would need to be addressed, and solvent-based delivery vehicles are one approach for doing so (Fu et al., 2009). Nanoparticles are often used in solvent-based delivery vehicles because they allow for controlled drug release to happen over time and with a predictable profile.

Albumin (molecular weight: 67 kDa, melting point: approximately 62 °C) is the most abundant human plasma protein. Because it is stable at physiological conditions, readily soluble, nontoxic, and nonimmunogenic, albumin is a commonly used drug delivery vehicle (Atala and Lanza, 2002; Michnik et al., 2006). For example, nanoparticle albumin-bound paclitaxil was approved by the Food and Drug Administration in 2006 to treat metastatic breast cancer (Fu et al., 2009). The molecular structure of albumin (FIGURE 1) consists of six subdomains and six sites for binding fatty acids (Pulla Reddy et al., 1999; Sugio et al., 1999). These sites can serve as pockets that bind hydrophobic

drugs such as curcumin, allowing them to become solubilized and protecting them from degradation, improving their bioavailability. Albumin has been shown to bind curcumin with 2:1 stoichiometry (Pulla Reddy et al., 1999).

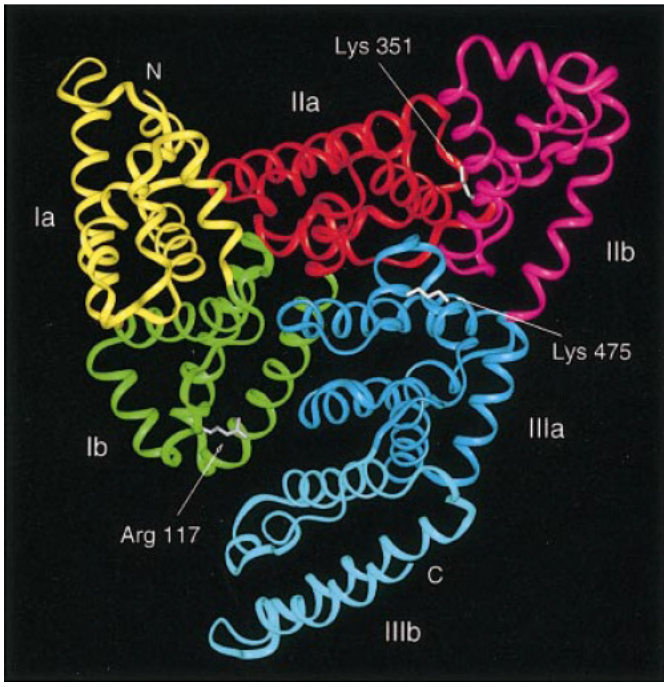


FIGURE 1 - Schematic drawing of HSA (Sugio et al., 1999)

Techniques such as emulsification, desolvation, and thermal gelation have all been used to produce albumin nanoparticles (Elzoghby et al., 2012). Spray drying, another technique, is commonly used in the pharmaceutical industry to produce dry particles from liquids (FIGURE 2). Spray drying can be performed using water as the solvent, making it an advantageous approach. When spray drying with a Nano Spray Dryer, a piezoelectric actuator is driven at an ultrasonic frequency, generating vibrations that direct the injection of the liquid phase into an array of micron-sized holes. These holes form consistently-sized droplets that fall down a column under high pressures, quickly drying

the droplets into dry particles. These particles are collected at the bottom of the column using an electrostatic precipitator. The method of spray drying is advantageous for albumin-based drug delivery because it is able to make compact particles with reproducible size, surface morphology, and release characteristics (Elzoghby et al., 2012).

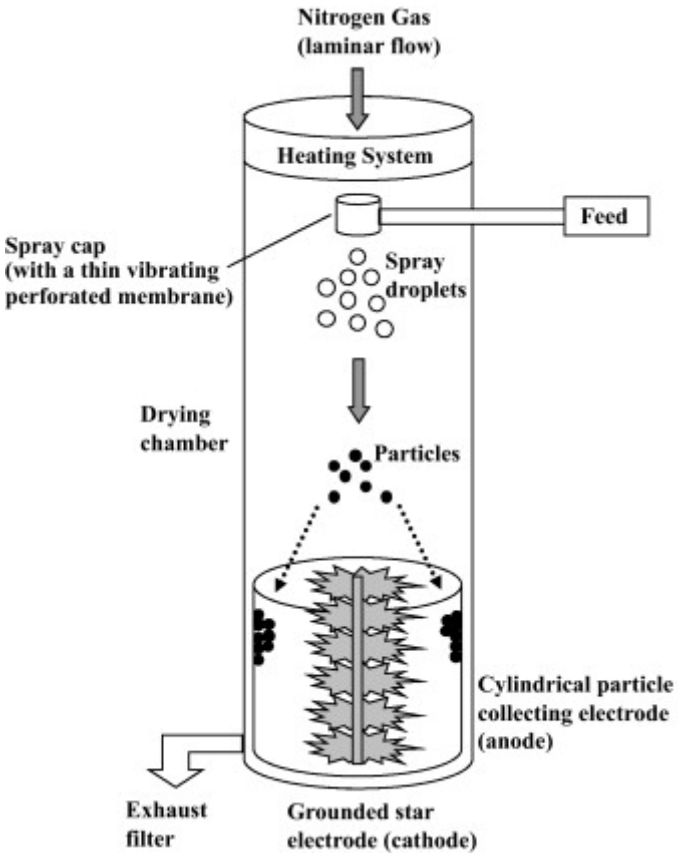


FIGURE 2 - Schematic of spray drying process (Lee et al., 2011)

B. Previous Work

Initial experiments were conducted to optimize curcumin solubility by binding it to various forms of albumin (CEW, BSA, and FAF BSA). Each type of albumin was examined at various concentrations (5%, 1%, and 0.1% w/v) in DI water. With each type of albumin, the increasing albumin concentrations resulted in increasing solubility of curcumin (FIGURE 3). After comparing the three types of albumin, statistical analysis showed that FAF BSA was able to solubilize the most curcumin.

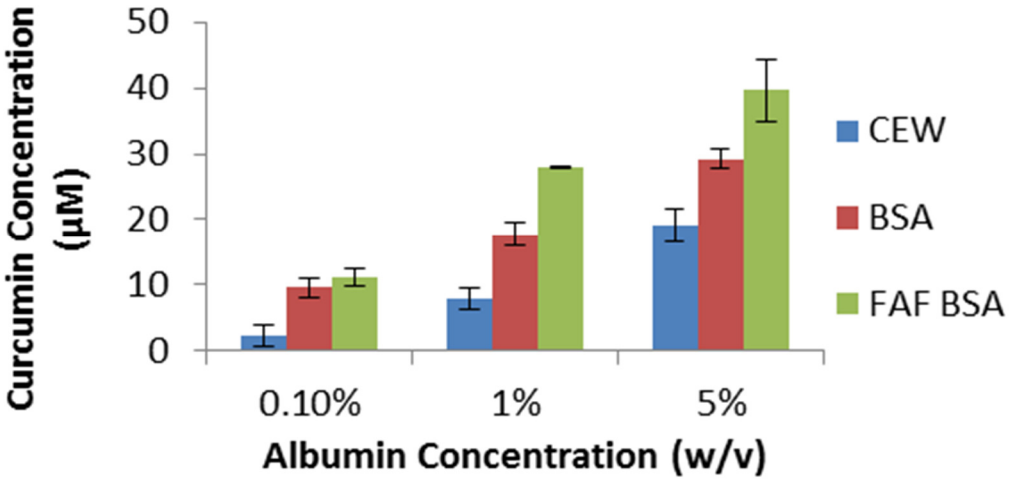


FIGURE 3 - Curcumin binding to CEW, BSA, and FAF BSA at various albumin concentrations.

For spray drying studies, HSA was used given the therapeutic applications of albumin-curcumin particles in humans. Since FAF BSA was shown to bind more curcumin than BSA, FAF HSA was the type of albumin selected for particle fabrication. FAF HSA-curcumin solutions were made with various albumin concentrations (0.1%, 0.5%, and 1% w/v) in DI water and then spray dried to form particles with a Buchi Nano

B-90 spray dryer (head temperature of 120 ºC, pressure of 45 hPa, drying gas flow rate of 150 L/min, peristaltic pump setting of 1, and mesh containing 4µm holes). To aid in particle formation, T20 (0.05% v/v) was added to the FAF HSA solutions prior to mixing with curcumin (Lee et al., 2011).

With increased FAF HSA concentrations, there was an increase in the average particle size. By using dynamic light scattering, it was determined that the average particle size ranged from 369 ± 180 nm for 0.1% FAF HSA-curcumin particles to 481 ± 200 nm for 1% FAF HSA-curcumin particles. The 0.5% FAF HSA concentration was chosen as the concentration to produce spray dried particles that balanced smaller particle size with more curcumin binding. The T20 addition to 0.5% FAF HSA-curcumin produced particles with a more smooth and spherical morphology as shown in the scanning electron microscope images in FIGURE 4.

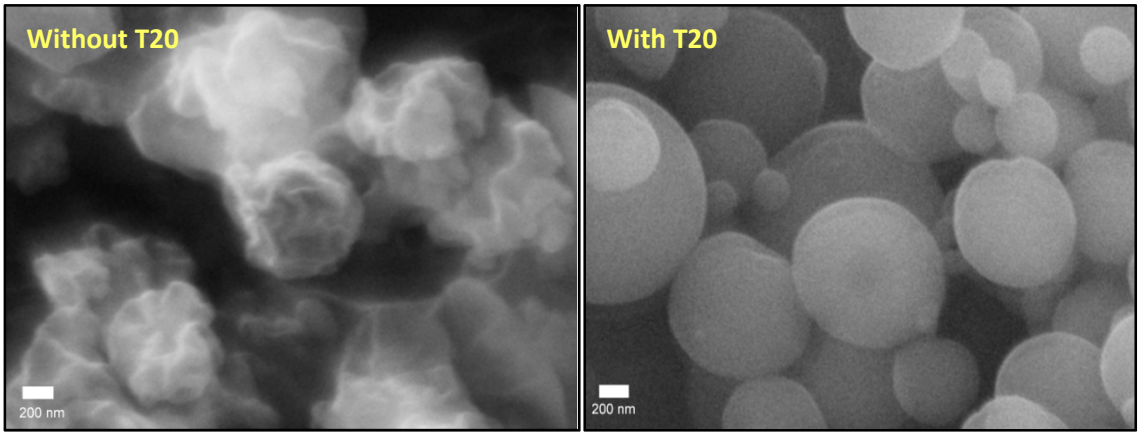


FIGURE 4 - Scanning electron microscope images of 0.5% FAF HSA-curcumin particles without and with 0.05% (v/v) T20 (Zeiss Evo, 20 kV, SE1 detector, sputter coated with gold/palladium for five minutes).

Curcumin release from FAF HSA particles was examined over time and the release profile best fit a first-order ($C_t/C_{inf} = 1 - e^{-kt}$, t = time) release. The protocol was adapted from Jithan et al., 2011. The release of curcumin from 2 mg/ml of 0.5% FAF HSA-curcumin particles reached a saturation level in approximately 1 hour and followed the first-order model of $C_t/C_{inf} = 1 - e^{-0.055t}$.

C. Albumin Crosslinking

Spray drying particles of albumin with bound curcumin allows for the production of particles with release characteristics that can be manipulated by using various production parameters or solution composition. The crosslinking of albumin was explored as a means for prolonging the release of bound curcumin because crosslinked albumin particles may need more time to solubilize and release curcumin. Additionally, the crosslinks on these albumin particles may extend the time needed for enzymatic breakdown once the particles enter a cell, prolonging the release of bound curcumin.

Three main crosslinking agents were considered – glutaraldehyde, BS^3, and EDC. These three crosslinking agents were initially chosen because of their solubility in water. Glutaraldehyde is commonly used as a fixative because of its aggressive protein crosslinking ability (Migneault et al., 2004). Though effective, glutaraldehyde has been classified as a skin, eye, and respiratory irritant (National Toxicology Program, 1999). BS^3 is a homobifunctional crosslinking agent that reacts with primary amines such as those found on lysine residues or the N-terminus of a polypeptide (Huang et al., 2004). It can perform its crosslinking procedure at physiologic conditions, but its cytotoxicity is still unknown. EDC is a zero-length crosslinker that crosslinks carboxylic acids to

primary amines. Thus, there are 187 potential EDC crosslinking sites (lysine, arginine, glutamic acid, and aspartic acid residues, one polypeptide N-terminus, and one polypeptide C-terminus) per molecule of HSA, however there are two lysines located on the molecular surface (Sugio et al., 1999). Additionally, no significant cytoxicity has been observed with EDC crosslinking (Park et al., 2002). Sulfo-NHS is also water-soluble and is often used in conjunction with EDC to improve its crosslinking ability by creating stable amine-reactive intermediates (FIGURE 5) (Hermanson, 2013). The advantages of the EDC/sulfo-NHS mechanism made it an ideal method to crosslink albumin for spray dried particle production. The crosslinking of albumin with EDC has not been previously explored for spray dried particles.

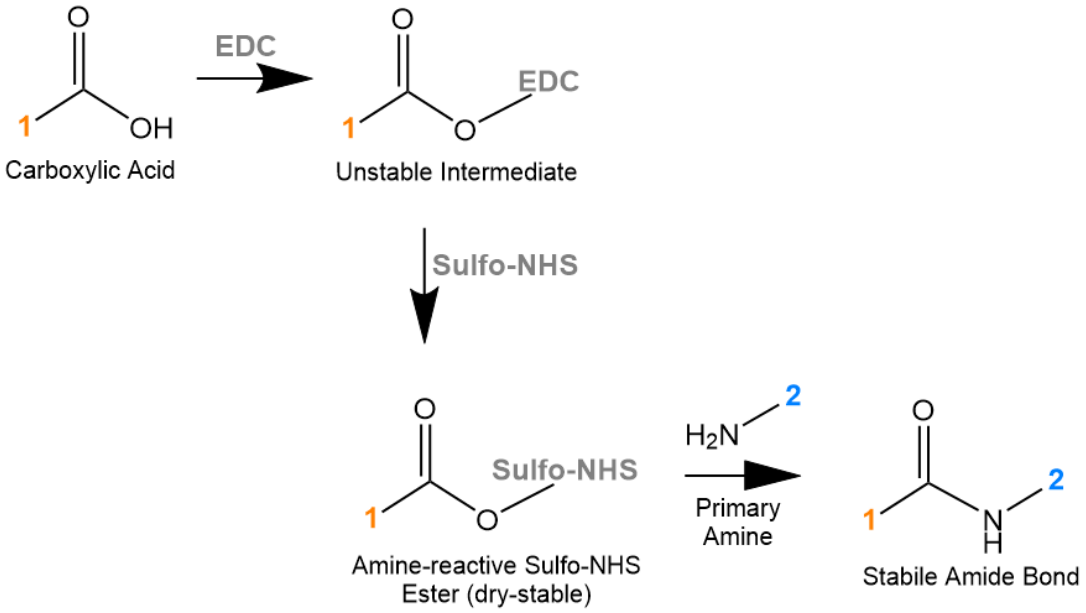


FIGURE 5 - Crosslinking mechanism for EDC with sulfo-NHS (Hayworth, 2014)

D. Objective

The objective of this was work was to alter the release profile of curcumin from spray dried albumin particles by crosslinking albumin with EDC and sulfo-NHS. To achieve this goal, this work covalently crosslinked albumin using EDC and sulfo NHS, spray dried the crosslinked albumin to form particles, and characterized particle size, morphology, curcumin loading capacity, and release kinetics.

II. METHODS

A. Crosslinking Albumin with Glutaraldehyde

Initially, 0.5% (w/v) BSA in DI water was crosslinked using glutaraldehyde at 100:1 and 15:1 glutaraldehyde:BSA molar ratios (Fuguet et al., 2007). Crosslinking was conducted on an orbital shaker for 24 hours at room temperature (20°C – 25°C) in a 50 mL centrifuge tube. The solution was dialyzed in DI water for 24 hours to removed excess glutaraldehyde.

B. Crosslinking Albumin with EDC and Sulfo-NHS

A second crosslinking method was also explored. The crosslinking protocol was adapted from Bioconjugate Techniques (Hermanson, 2013), and it crosslinked FAF HSA (SeraCare Life Sciences) using EDC (Acros Organics) and sulfo-NHS (Aldrich Chemistry). For this process, a final concentration of 0.01M EDC in DI water and 5mM sulfo-NHS in DI water were used to crosslink 0.5% (w/v) FAF HSA in DI water (7.5×10^{-5} M). This solution was deemed a "1x" level of crosslinking based on the amount of EDC that was added. An EDC concentration of 0.1 M was used to crosslink at the 10x level (n = 1). The crosslinking process was conducted for 24 hours on an orbital shaker at room temperature in a 50 mL centrifuge tube. Based on the molar ratio of EDC to FAF HAS for the 1x conditions listed above, 134 crosslinks could be theoretically formed.

Upon completion of crosslinking, by products of the crosslinking reactions and excess crosslinking reagents were removed via dialysis using 3500 MWCO dialysis tubing (Spectra/Por 7 Dialysis Membrane, Pre-treated RC Tubing). Prior to dialysis, the

membrane was rinsed thoroughly with DI water and allowed to soak in DI water for thirty minutes. Fifteen minutes into this soaking period, the DI water was replaced with fresh DI water. The crosslinked FAF HSA solution was dialyzed in 4 L DI water for 24 hours at room temperature. After the first 24 hours of dialysis, the DI water was replaced with 4 L of fresh DI water. The dialysis was continued for another 24 hours.

C. Particle Fabrication by Spray Drying

Spray drying was performed using the Buchi Nano Spray Dyer B-90 at a head temperature of 120 °C, drying gas flow rate of 150 L/min (Lee et al., 2011), peristaltic pump setting of 2 (pump rotation at double the standard speed), and mesh containing 4µm holes . The sample being sprayed was kept on ice and covered in foil. The spray dried particles were collected by scraping them off of the collecting drum onto weigh paper in a chemical hood. These particles were placed in a centrifuge tube wrapped in foil and stored at -20 °C for 1 day. The same conditions were used for spray drying all conditions unless noted.

D. Confirmation of Albumin Crosslinking

The crosslinking of FAF HSA with EDC and sulfo-NHS was confirmed using two different methods. The first method used to confirm FAF HSA crosslinking was native PAGE. This method used an electrical charge to pull proteins through a polyacrylamide gel achieving separation based on molecular weight and geometry (both would be altered by crosslinking albumin). The Bio-Rad Mini-PROTEAN Tetra System was used with 7.5% precast polyacrylamide gels. Buffers were made according to the adapted

formulations provided in TABLE II from Bio-Rad Laboratories, Inc. Solutions of FAF HSA (n = 4) and 1x FAF HSA (n = 4) in DI water were made. The gel was loaded with 8 µg of regular FAF HSA or 1x FAF HSA (as determined by absorbance at λ = 280 nm) per well at a 1:1 volume ratio with the Native PAGE sample buffer for a total loading solution volume of 40 µL. Additionally, one lane was loaded with 9µL of the Bio-Rad Precision Plus Unstained Protein Standard, 1 µL of β-mercaptoethanol (Sigma), and 30 µL of Native PAGE running buffer. Lanes not used with samples were loaded with 40 µL of Native PAGE running buffer. The loaded gel was run in the Tetra System at 100 V and 4 °C for 1.5 hours with the Bio-Rad PowerPac Basic serving as the voltage source. Upon completion of this run, the gel was removed from its casing and stained using Coomassie Brilliant Blue R-250 Staining Solution (Bio-Rad) at room temperature while on a rocker for 3 hours. The polyacrylamide gel was then exposed to Coomassie Brilliant Blue R-250 Destaining Solution (Bio-Rad) at room temperature with rocking for 12 hours. A digital image of the gel was captured. Destaining continued for another 13 days. On day 14, another digital image of the gel was captured.

TABLE II

NATIVE PAGE BUFFER FORMULATIONS

Native PAGE Running Buffer	1.5g Trizma Base (Sigma Life Science) 7.2g Glycine (Sigma Life Science) 500mL DI Water	
Native PAGE Sample Buffer	0.5mL of 0.5M Tris-HCl, pH 6.8 1.1mL Glycerol (Sigma-Aldrich) 0.04mL of 1% Bromophenol Blue 1.86mL DI Water	
0.5M Tris-HCl	6.06g Trizma Base ~60mL DI Water Total Volume = 100mL	Adjust to pH 6.8 with HCl (Sigma). Make to 100mL with DI Water.

1% Bromophenol Blue	100mg Bromophenol Blue (Sigma-Aldrich) 10mL DI Water	

The second method employed DSC (TA Instruments, Auto Q20) to compare the melting points of spray dried FAF HSA between its uncrosslinked and 1x crosslinked forms. DSC reports the heat flow (W/g) needed to maintain a steady increase in temperature for a sample of known mass. The DSC was calibrated with indium across a temperature range of 20 °C to 100 °C using a ramp rate of 2 °C/min and nitrogen flow rate of 50 mL/min. The FAF HSA samples were prepared in solution with DI water, and as spray dried particles. The samples were placed in aluminum pans (TA Instruments, Standard Pan and Lid) and heated in the DSC from 20 °C to 100 °C using a ramp rate of 2 °C/min and nitrogen flow rate of 50 mL/min. An empty aluminum pan was simultaneously heated in the DSC as a reference. Melting points were determined by finding the endothermic peaks of the DSC curves (heat flow v. temperature) over the range of 20 °C to 100 °C using the Q90 Universal Analysis software (TA Instruments). For the DSC melting point results from spray dried FAF HSA and 1x FAF HSA, one sample was spray dried for each condition. Five aliquots of the FAF HSA sample and four aliquots of the 1x FAF HSA sample were measured. Results are reported as average ± SEM °C. Statistical significance was determined by conducting a t-test (Microsoft Excel) with unequal variances and $\alpha = 0.05$ on these measurements.

E. Curcumin Binding

For the testing conditions with curcumin, 2.5 mg/ml curcumin (Sabinsa) and 0.05% (v/v) T20 (Sigma) were added to the 1x FAF HSA in DI water after dialysis was

complete. Due to curcumin's sensitivity to light, the solution was wrapped in aluminum foil once curcumin was added. The solution was placed on an orbital shaker for 24 hours at 4 °C. Curcumin was bound to FAF HSA in the presence of T20 using this same procedure.

After curcumin was allowed to bind to 1x FAF HSA-T20 and FAF HSA-T20, excess unbound curcumin was removed via centrifugation (Beckman Coulter Allegra 6R Centrifuge). The solutions were centrifuged at 3750 rpm (3210 x g based on Equation 1; rotational radius, r = 20.4 cm) and 4 °C for 20 minutes. The unbound curcumin formed a pellet during this centrifugation. The supernatants were removed and centrifuged again at 3210 x g and 4 °C for another 20 minutes. The supernatant solutions were wrapped in aluminum foil.

$$\text{g Force} = (\text{rpm})^2 \times 1.118 \times 10^{-5} \times \text{r} \quad (1)$$

F. Quantification of Curcumin Bound to Albumin

A standard curve was generated with curcumin initially dissolved in DMSO (Sigma Life Science) at a concentration of 5mM. UV-vis absorbance measurements were collected of a 0.125 mM curcumin solution that was created by diluting the 5mM curcumin in 0.5% FAF HSA in DI water with 0.05% T20. This solution was serially diluted in 0.5% FAF HSA in DI water with 0.05% T20 and vortexed to generate the standard curve. These measurements were blanked with a solution containing 50 µL of DMSO in 1.95 mL of 0.5% FAF HSA in DI water with 0.05% T20.

To determine the concentration of bound curcumin, supernatant absorbance was quantified using UV-visible spectroscopy (Thermo Scientific NanoDrop 2000c Spectrophotometer) at a wavelength of $\lambda = 425$ nm (the peak absorbance of curcumin) for solutions of FAF HSA-T20-curcumin (n = 3) and 1x FAF HSA-T20-curcumin (n = 3). Results are reported as average ± SEM μM curcumin. Statistical significance was determined by conducting a t-test (Microsoft Excel) with unequal variances and $\alpha = 0.05$ on the absorbance readings for the FAF HSA-T20-curcumin and 1x FAF HSA-T20-curcumin solutions.

Measurements were also collected for negative control conditions of FAF HSA-T20 (n =1) and 1x FAF HSA-T20 (n = 1) in DI water. Three measurements were taken for each of these samples, and the reported values for curcumin binding are the average ± SEM μM curcumin of these three measurements. Molar ratios of bound curcumin to FAF HSA were calculated using Equation 2.

$$x = \frac{(\text{bound curcumin in } \mu M)(10^{-6})}{\left(5\frac{g}{L} \text{ of FAF HSA}\right)\left(\frac{1 mol}{67{,}000\ g}\right)};\ \text{Bound Curcumin:FAF HSA} = x{:}1 \qquad (2)$$

G. Characterization of Particles

Particle size and morphology were characterized using scanning electron microscopy for FAF HSA-T20-curcumin (n = 3) and 1x FAF HSA-T20-curcumin (n = 3) particles. Particles were sprinkled onto carbon tape on pegs that were sputter coated with gold/palladium at 0.1 torr and 5mA for 4 minutes. Particles were visualized with a Zeiss Supra 35VP scanning electron microscope using a secondary electron detector with EHT = 2.0 kV. Three images were captured for each sample. Particle size was measured using

ImageJ software developed by the National Institutes of Health (Rasband, 1997). The scale bar on the original image was used to calibrate the dimensions of the image pixels. The long axis of each particle whose full diameter was visible was measured and considered the particle diameter. Particles with smooth and spherical morphology were counted for each image, and these counts were normalized to the magnification of 54703 X (27.5 μm^2 image area). Results are reported as average ± SEM. Statistical significance was determined by conducting a t-test (Microsoft Excel) with unequal variances and α = 0.05 on the size and morphology measurements for FAF HSA-T20-curcumin and 1x FAF HSA-T20-curcumin particles. Images and measurements were also collected for negative control conditions of FAF HSA-T20 (n =1), 1x FAF HSA (n = 1) and 1x FAF HSA-T20 (n = 1) particles. Three measurements were taken for each of these samples, and the reported values are the average ± SEM of these three measurements.

H. Profiling Curcumin Release

Curcumin release from FAF HSA-T20-curcumin (n = 3) and 1x FAF HSA-T20-curcumin (n = 3) spray dried particles over time was profiled via differential extraction and UV-visible spectroscopy (Jithan et al., 2011; O'Toole et al., 2012). For differential extraction, the particles were added to 1% L-ascorbic acid (Sigma) in PBS (Fisher BioReagents) to make the aqueous layer. The solution was mixed via inversion of the tube, and then an equivalent volume of 0.1% BHT (Aldrich) in octanol (Sigma-Aldrich) was gently added to serve as the organic layer. Particles were added at a concentration of 2 mg/mL based on the total volume of the aqueous and organic layers. Upon release from the particles, curcumin was pulled into the organic layer because of its hydrophobicity.

The absorbance of this organic layer was measured by using UV-visible spectroscopy (Thermo Scientific NanoDrop 2000c Spectrophotometer) at a wavelength of $\lambda = 425$ nm. Spectral scans indicated that this was the peak absorbance for curcumin in octanol. The release study was considered complete once the absorbance readings from three consecutive time points confirmed a plateau for curcumin release. Curcumin concentration was calculated using a standard curve of curcumin in 0.1% BHT in octanol (2500 mM to 2.5 mM). Each solution was vortexed prior to collecting the absorbance readings. All readings were blanked with 0.1% BHT in octanol. As negative controls, release studies were also conducted with FAF HSA-T20 (n = 1) and 1x FAF HSA-T20 (n = 1) particles.

Raw curcumin release concentrations were converted to ratios of total released curcumin (C_t/C_{inf}). For each measurement, the curcumin concentrations of the final three time points were averaged and represented maximum curcumin release (C_{inf}). Curcumin concentrations for the earlier time points (C_t) were divided by this C_{inf}. C_t/C_{inf} became the dependent variable used to fit curcumin release profiles to the first-order mathematical model. First-order models were generated using Minitab 16 statistical analysis software. SSE was used as an indicator of fit for the first-order model. Statistical significance was determined by conducting a t-test with unequal variances (Microsoft Excel, $\alpha = 0.05$) on the sample k terms output for the first-order models of curcumin release from FAF HSA-T20-curcumin (n = 3) and 1x FAF HSA-T20-curcumin (n = 3) particles.

I. Additional Experiments

Additional studies were conducted to explore methods for optimizing the crosslinked FAF HSA spray dried particles. 1x FAF HSA (n = 1) in DI water was made with double the concentration of T20 (0.1%) to observe changes in particle morphology via scanning electron microscopy. Additionally, spray drying was attempted with a 2.5x FAF HSA-T20 solution (n = 1).

III. RESULTS AND DISCUSSION

A. Crosslinking Albumin with Glutaraldehyde

BSA crosslinked with glutaraldehyde at 100:1 glutaraldehyde:BSA formed a gel-like substance that could not be spray dried through the small diameter (4µm) holes in the spray drying mesh without causing clogging. The 15:1 glutaraldehyde:BSA appeared to have a lower viscosity that perhaps could have been spray dried. However, glutaraldehyde toxicity remained a concern for the therapeutic potential of spray dried particles made from this crosslinking technique (Atala and Lanza, 2002). An alternative method for crosslinking was simultaneously pursued to overcome some of the hurdles associated with the glutaraldehyde technique.

B. Crosslinking Albumin with EDC and Sulfo-NHS

FAF HSA was crosslinked in DI water at various levels ranging from 1x to 10x based on the concentration of EDC used for the crosslinking. It was found that the 10x level of crosslinking produced a gel-like solution of crosslinked FAF HSA that was not able to be spray dried. The 1x crosslinked FAF HSA was able to be spray dried without clogging the spray mesh. Unless noted differently, the 1x condition was used in the subsequent studies to confirm FAF HSA crosslinking, characterize the particles, and profile curcumin release from the particles.

C. Confirmation of Albumin Crosslinking

1. Native PAGE

Crosslinking of FAF HSA with EDC and sulfo-NHS was confirmed using Native PAGE. The digital image of the gel during the destaining process at the 12 hour time point (FIGURE 6A) showed the bands of FAF HSA near the 75 - 50 kDa range with some smearing. Destaining was continued to reduce the smearing in the FAF HSA samples. The digital image of the destained gel at the 14 day time point (FIGURE 6B) confirmed that the bands of FAF HSA were in the 75 - 50 kDa range. The gel lanes loaded with 1x FAF HSA contained multiple bands at molecular weights above the molecular weight of FAF HSA. These bands represented multiple FAF HSA units being crosslinked to one another, forming larger globules of FAF HSA that had an altered geometry and increased molecular weight. The difference in protein band profiles for the FAF HSA and 1x FAF HSA confirmed that FAF HSA was crosslinked.

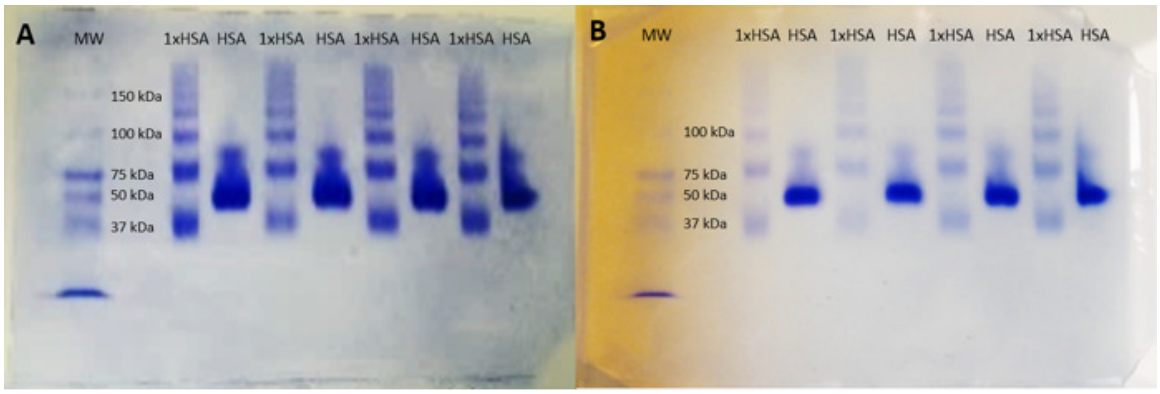


FIGURE 6 - Coomassie-stained native PAGE gel after (A) 12 hours and (B) 14 days of exposure to destain solution. From left to right in each image, the first lane was loaded with the molecular weight protein standard (MW). The next lane was loaded with running buffer only. The third lane was loaded with 1x FAF HSA, and the fourth lane was loaded

with FAF HSA. The conditions for the third and fourth lanes were repeated for the remaining lanes.

2. DSC

The melting point of FAF HSA in DI water was compared to the melting point of 1x FAF HSA in DI water. These results did not consistently generate distinguishable endothermic peaks. Most likely, there was too low a concentration of FAF HSA present in the solutions to produce repeatable results. Lyophilized FAF HSA from the supplier generated endothermic peaks at 72.74 ± 2.68 ºC. The lyophilized 1x FAF HSA gave inconsistent results.

Given that the final product being explored was FAF HSA in its spray dried form, melting points for spray dried FAF HSA particles were compared to the melting points of spray dried 1x FAF HSA particles using DSC. Reproducible and distinguishable endothermic peaks were identified (TABLE III). The 1x FAF HSA particles had a melting point of 63.82 ± 2.34 ºC, which was significantly higher ($p = 0.0128$) than the melting point of 53.60 ± 1.35 ºC for the FAF HSA particles. With the additional bonds formed via crosslinking, an increase in the amount of energy needed to break these bonds and melt crosslinked FAF HSA was expected. Consequently, the higher melting point observed for 1x FAF HSA particles supported the Native PAGE conclusion that FAF HSA was crosslinked with the EDC/sulfo-NHS. Representative DSC curves are shown in FIGURE 7 to illustrate the shift in melting point between FAF HSA particles and 1x FAF HSA particles.

TABLE III

DSC RESULTS FOR FAF HSA PARTICLES V. 1x FAF HSA PARTICLES

	Melting Point	
	Raw Data (ºC)	**average ± SEM (ºC)**
FAF HSA	55.60, 51.03, 57.76, 50.76, 52.87[1]	53.60 ± 1.35 *
1x FAF HSA	57.68, 64.79, 63.82[2], 69.00	63.82 ± 2.34 *

[1,2]The DSC curves for these measurements are depicted in FIGURE 7.

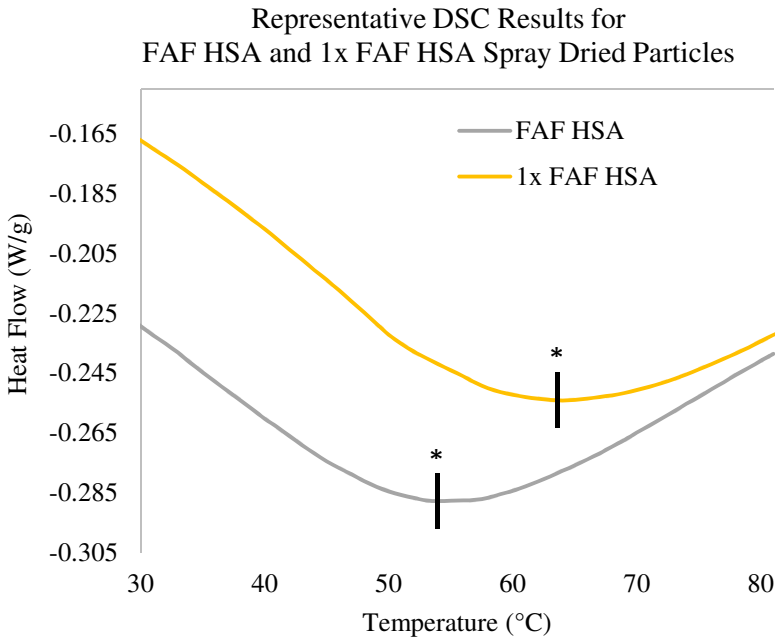


FIGURE 7 - Representative DSC endothermic peak of 1x FAF HSA was shifted to the right from FAF HSA, which indicated a higher melting temperature for 1x FAF HSA.

D. Quantification of Curcumin Bound to Albumin

The concentration of curcumin bound to FAF HSA-T20 in DI water as measured prior to spray drying was 115.551 ± 6.918 µM. This was calculated to be a molar ratio of approximately 1.55:1 for curcumin:FAF HSA. The concentration of curcumin bound to 1x FAF HSA-T20 in DI water as measured prior to spray drying was 114.316 ± 9.970 µM. This was calculated to be a molar ratio of approximately 1.53:1 for curcumin:FAF HSA. As negative controls, FAF HSA-T20 and 1x FAF HSA-T20 exhibited negligible readings at 425 nm. Even though FAF HSA was crosslinked, these crosslinks did not significantly reduce curcumin binding ($p = 0.9185$) to the FAF HSA (FIGURE 8).

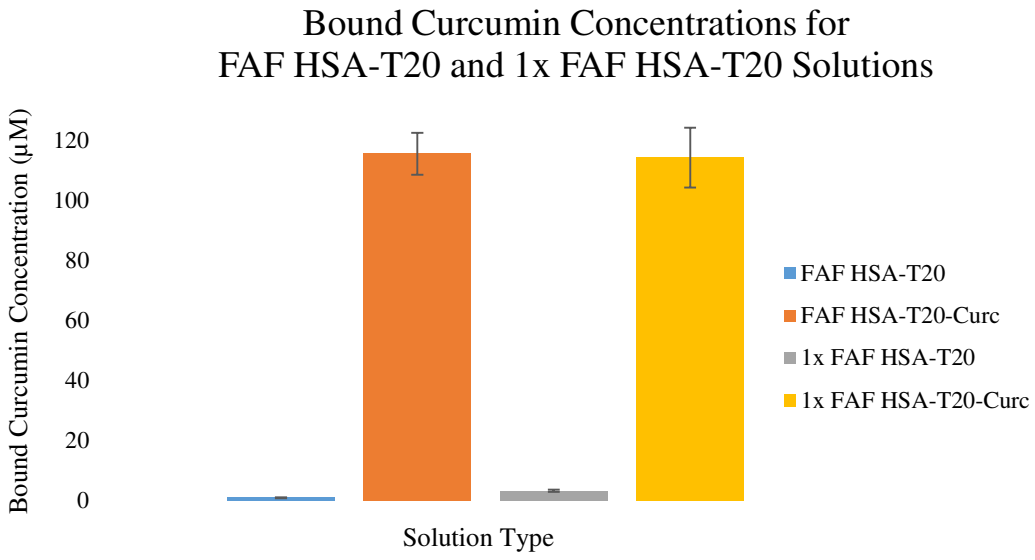


FIGURE 8 - Concentrations of curcumin bound to FAF HSA-T20 and 1x FAF HSA-T20 in DI water.

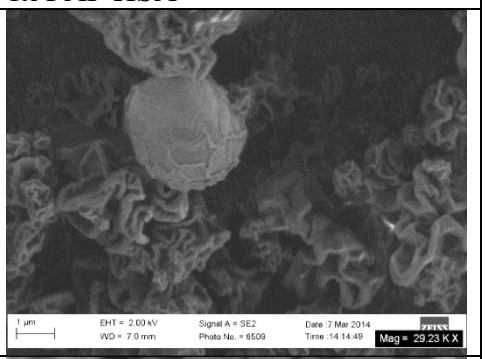
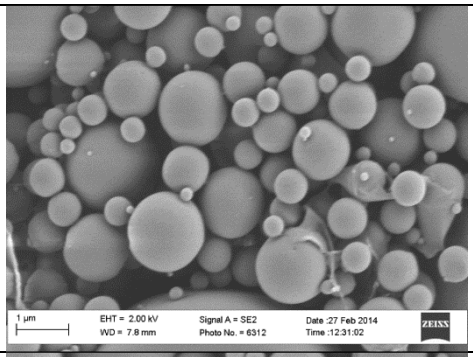
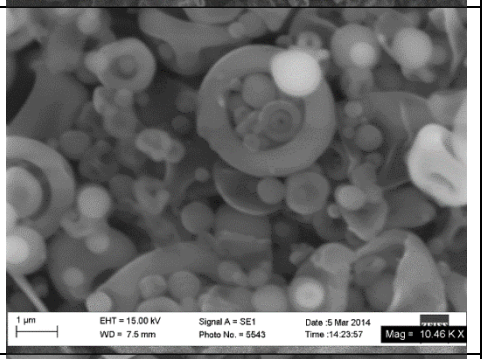
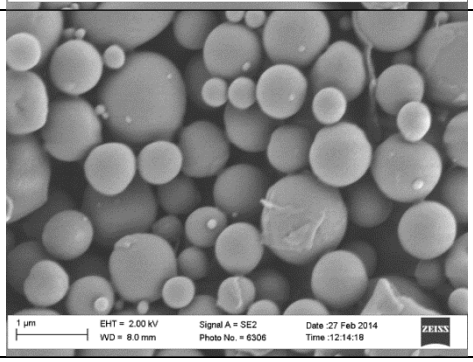
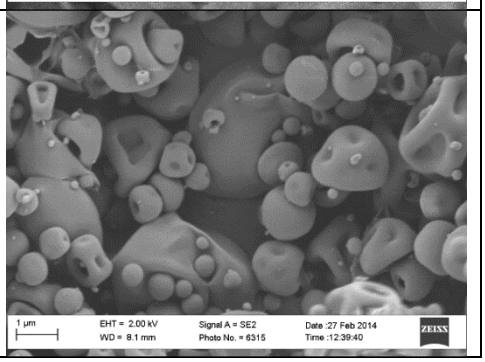
Characterization of Particles

1. Morphology

Scanning electron microscopy images of the various spray dried particles are shown in TABLE IV. FAF HSA-T20 and FAF HSA-T20-curcumin particles showed spherical and smooth morphologies. Since the release of curcumin from spray dried albumin particles is affected by variations in particle shape and surface morphology, this spherical shape and uniform surface morphology was desirable. The 1x FAF HSA-T20 particles were not consistently spherical and smooth as observed with the FAF HSA-T20 particles produced under the same conditions. The 1x FAF HSA particles showed a wrinkled morphology that became smoother in the presence of T20 (this was also seen with FAF HSA, Lee et al., 2011). The counts of particles with smooth and spherical morphology per 27.5 μm^2 area for FAF HSA-T20-curcumin were (55.5 ± 9.8) and 1x FAF HSA-T20-curcumin (14.0 ± 2.1) (TABLE V). Even though differences in morphology were observed between these conditions, the difference was not statistically significant ($p = 0.0541$).

TABLE IV

SCANNING ELECTRON MICROSCOPY IMAGES OF SPRAY DRIED PARTICLES

	FAF HSA	1x FAF HSA
No T20, No Curcumin	-----	
T20, No Curcumin		
T20, Curcumin		

2. Size

The results for spray dried particle diameters are outlined in TABLE V. Theoretical calculations report the minimum viable diameter for human capillaries to be approximately 4 µm (Freitas, 1999) – an important upper size limit for the potential intravenous administration of these particles. The observed particle diameters fell well

below this upper size limit. No statistical significance was observed between diameters for the FAF HSA-T20-curcumin particles and the 1x FAF HSA-T20-curcumin particles. Therefore, crosslinking 0.5% FAF HSA with EDC and sulfo-NHS did not result in a significant change of particle diameter ($p = 0.9889$).

TABLE V

SIZE AND MORPHOLOGY MEASUREMENTS FOR SPRAY DRIED PARTICLES.

	Smooth and Spherical Particles	**Diameter (nm)**
FAF HSA-T20	56.8 ± 7.2	618.552 ± 14.837
FAF HSA-T20-Curcumin	55.5 ± 9.8	713.994 ± 25.723
1x FAF HSA-T20	4.4 ± 1.4	742.095 ± 43.087
1x FAF HSA-T20-Curcumin	14.0 ± 2.1	712.938 ± 65.292

E. Profiling Curcumin Release

1. Raw Curcumin Release Data

The absorbance spectral scans shown in FIGURE 9 confirmed that curcumin's peak absorbance occurred at $\lambda = 425$ nm for these release studies. Curcumin release data for FAF HSA-T20 versus FAF HSA-T20-curcumin particles is shown in FIGURE 10. The C_{inf} for the FAF HSA-T20-curcumin particles was 137.796 ± 7.330 μM, and the C_{inf} for the 1x FAF HSA-T20-curcumin particles was 146.957 ± 5.414 μM. As negative controls, FAF HSA-T20 and 1x FAF HSA-T20 particles showed negligible absorbance at 425nm.

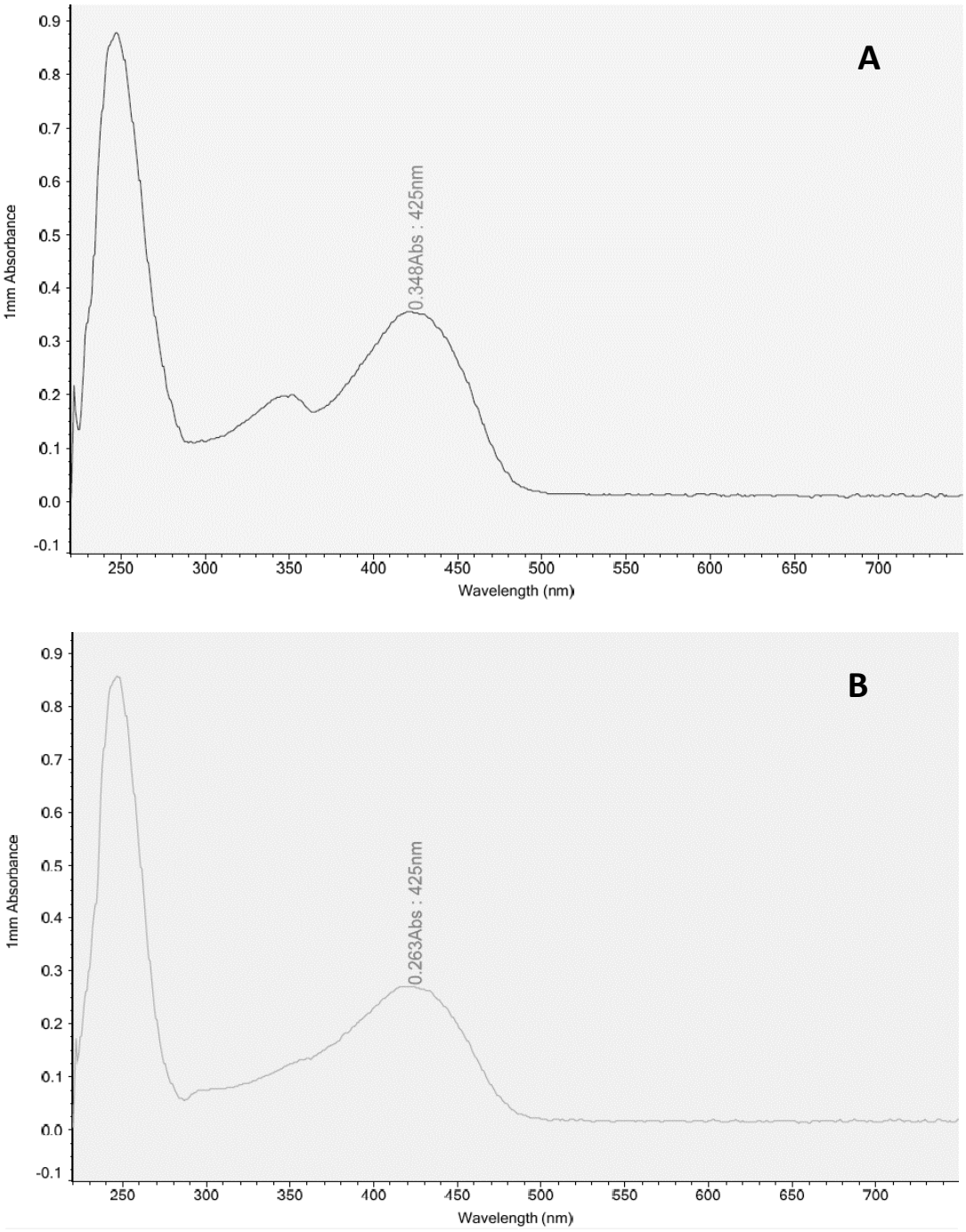


FIGURE 9 - Representative spectral scans for absorbance of organic layer from release study of FAF HSA-T20-curcumin (A) and 1x FAF HSA-T20-curcumin (B) spray dried particles at 180 minutes.

2. Fitting of Curcumin Release Model

FIGURE 10 shows an overlay of the average release profiles of FAF HSA-T20-curcumin and 1x FAF HSA-T20-curcumin particles for comparison. The k values determined from the first order fits to FAF HSA-T20-curcumin particles were 0.065 ± 0.003 and 1x FAF HSA-T20-curcumin particles was 0.049 ± 0.004. The release profile from 1x FAF HSA-T20-curcumin particles had a significantly ($p = 0.0339$) lower k than from FAF HSA-T20-curcumin particles, which implied a slower release.

FIGURE 11 shows the first-order models generated by Minitab software. Curcumin release from the FAF HSA-T20-curcumin particles had an SSE of 0.0006. Curcumin release from the 1x FAF HSA-T20-curcumin particles had an SSE of 0.0022. The low SSE values for both of these first-order models indicated that the first-order model was a good fit to the data and exploration of other release models was not needed.

Based on the first-order models that were generated, it took approximately 10.7 minutes for 50% of the maximum curcumin release to occur for the FAF HSA-T20-curcumin particles as compared to approximately 14.1 minutes for the 1x FAF HSA-T20-curcumin particles. It took approximately 46.1 minutes and 61.1 minutes for 95% of the maximum curcumin release to occur for the FAF HSA-T20-curcumin and 1x FAF HSA-T20-curcumin particles, respectively. This illustrates the slowed curcumin release from the crosslinked particles.

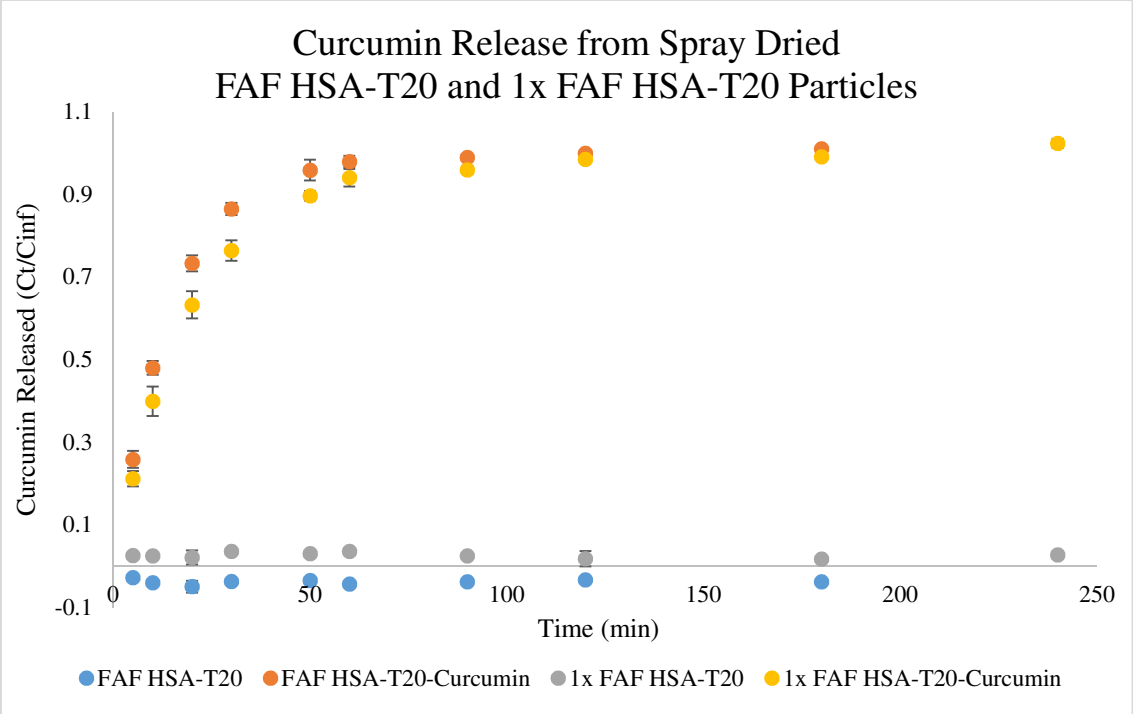


FIGURE 10 - Curcumin release data for FAF HSA-T20, FAF HSA-T20-curcumin, 1x FAF HSA-T20, and 1x FAF HSA-T20-curcumin spray dried particles.

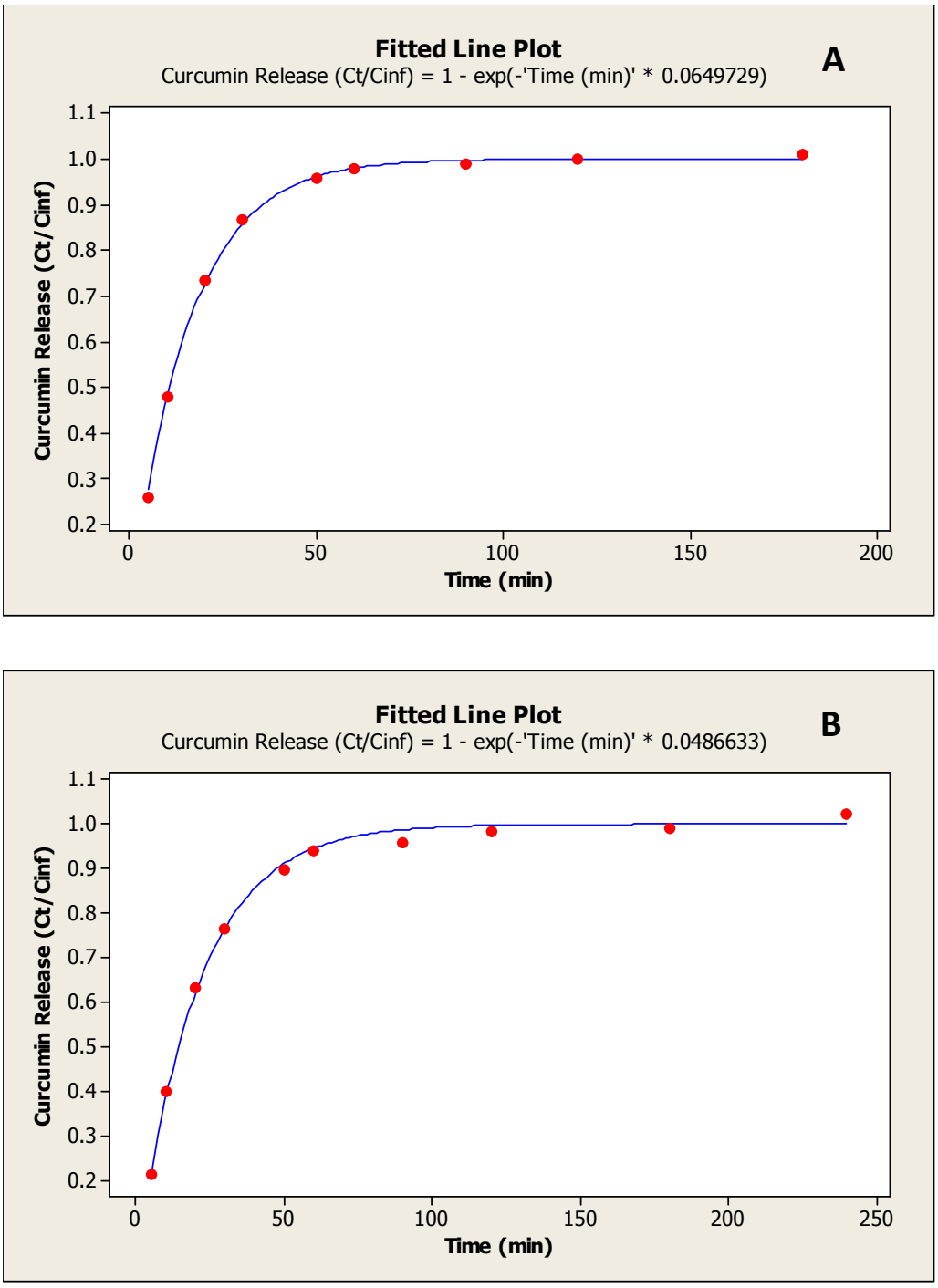


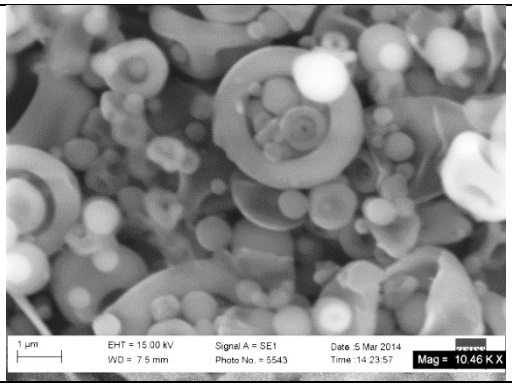
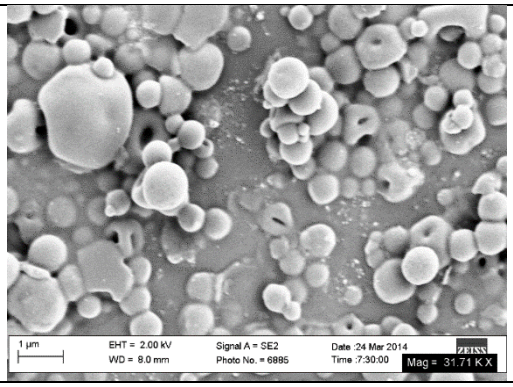
FIGURE 11 - Fitted line plot of first-order models for curcumin release from FAF HSA-T20-curcumin (A) and 1x FAF HSA-T20-curcumin (B) spray dried particles generated using Minitab software.

F. Additional Experiments

By doubling the T20 that was added to 1x FAF HSA, the production of more spherical and smooth spray dried particles was observed (TABLE VI). Spray drying of 2.5x FAF HSA-T20 was conducted without clogging of the spray mesh. Higher concentrations of T20 improved the morphology of 1x FAF HSA particles, and the feasibility of spray drying FAF HSA with increased levels of crosslinking was proven.

TABLE VI

SCANNING ELECTRON MICROSCOPY IMAGES OF 1X FAF HSA PARTICLES WITH 0.05% T20 AND 0.1% T20

1x FAF HSA-0.05% T20	1x FAF HSA-0.1% T20
	

IV. CONCLUSIONS, DISCUSSION, & RECOMMENDATIONS FOR FUTURE WORK

This work produced crosslinked albumin particles with altered curcumin release using the spray drying method. Crosslinking FAF HSA using EDC and sulfo-NHS was confirmed using native PAGE and DSC. The native PAGE results showed multiple protein bands for 1x FAF HSA as compared to the single band for uncrosslinked FAF HSA. The protein bands for 1x FAF HSA were associated with higher protein molecular weights than the single protein band for uncrosslinked FAF HSA, suggesting that multiple FAF HSA molecules had been crosslinked to each other. DSC results reported a significant increase of 10.22 °C in melting point for spray dried 1x FAF HSA particles as compared to uncrosslinked FAF HSA particles, further confirming the crosslinking method that was used.

Curcumin solubilization via binding to the 1x FAF HSA was not significantly altered by the formation of the crosslinks in albumin. Therefore the loading efficiencies of curcumin were the same for uncrosslinked and crosslinked albumin. The 1x FAF HSA was then spray dried to form particles that were 712.938 ± 65.292 nm in diameter. Crosslinking albumin did not alter the diameter of the particles formed by spray drying, but the morphology of the particles was different when albumin was crosslinked.

The morphology of 1x FAF HSA particles was smoother with the addition of 0.05% T20, but 1x FAF HSA-T20 and 1x FAF HSA-T20-curcumin particles did not exhibit the same consistent spherical morphology as their FAF HSA-T20 and FAF HSA-T20-curcumin counterparts. Counts for particles with smooth and spherical morphology were not statistically significant. Still, the difference in observed particle morphology (TABLE

IV) is of importance because it could contribute to altered curcumin release from 1x FAF HSA-T20-curcumin particles. No significant difference in particle diameter was observed for FAF HSA-T20-curcumin and 1x FAF HSA-T20-curcumin particles.

The release of curcumin from FAF HSA-T20-curcumin particles followed a first-order release profile with the equation $C_t/C_{inf} = 1\text{-}e^{-0.065t}$. The release of curcumin from 1x FAF HSA-T20-curcumin particles followed a first-order release profile with the equation $C_t/C_{inf} = 1\text{-}e^{-0.049t}$. Statistical significance was observed between these two release profiles with the curcumin release from 1x FAF HSA-T20-curcumin particles being slowed. Further extending curcumin release from FAF HSA particles should be explored by increasing the level of crosslinking. Crosslinking at a 2.5x level produced a solution that could be spray dried (while stirring constantly). The upper limit of crosslinking that is capable of being spray dried needs to be determined. Other techniques to form albumin nanoparticles such as electrospraying and others listed in the introduction chapter will need to be explored when the upper limit of crosslinking is reached.

The lower *k* value for the curcumin release from 1x FAF HSA-T20-curcumin particles indicated prolonged curcumin release from these particles as compared to the FAF HSA-T20-curcumin particles. In order to confirm that curcumin release is in fact delayed for EDC crosslinked FAF HSA particles, particle morphology must be improved to where smooth and spherical particles are consistently being produced via the spray drying process. Previously collected data showed that the addition of T20 aided in smooth albumin particle formation. Thus, one potential way to improve crosslinked particle morphology would be to increase the concentration of T20 used. Other

parameters that should be investigated include changing the pump setting to “normal” speed and reducing the gas flow rate on the Buchi B-90 Nano spray dryer.

This work shows that crosslinking albumin with EDC and sulfo-NHS can produce spray dried particles with slowed curcumin release. The cytotoxicity of the crosslinked particles may be tested on cells in the future to show that they are non-toxic. The bioactivity of curcumin released from crosslinked particles can be observed by testing the viability of cancer cells treated with these particles. Curcumin delivery via spray dried albumin particles shows promise and should continue to be explored.

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EDUCATION

M. Eng. Bioengineering — May 2014
J.B. Speed School of Engineering, University of Louisville, Louisville, KY

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Engineering High Honors, University Honors Scholar

EMPLOYMENT

European Space Agency Astronaut Center, Wyle Laboratories

Crew Medical Support Office Intern, Cologne, Germany — Jun 2014 - July 2014
Will contribute to the development of a novel rehabilitation device targeting lumbo-pelvic stabilizer muscles that experience deconditioning during long-duration space flight

National Aeronautics and Space Administration (NASA), Johnson Space Center

Student Ambassador — Feb 2014 - Present
Selected to join a cohort of high-achieving NASA interns that aim to engage and inspire the next generation of space explorers

Undergraduate Student Research Program Intern, Houston, TX — Jan 2013 - Apr 2013
Investigated cardiovascular physiology in space and its clinical implications during a dual-project internship with NASA's Cardiovascular Lab and Astronaut Occupational Health Program

Space Suit Assembly Engineering Intern, Houston, TX
Improved the design, development, assembly, and testing of prototype space suits for future deep space exploration missions — May 2012 - July 2012

University of Louisville

Student Researcher, Department of Bioengineering, Louisville, KY — May 2010 - Present
Completing work on NASA-funded drug delivery project to prevent radiation-induced cancer. This work has resulted in five poster presentation and has developed into my M.Eng. thesis.

Graduate Recruiter, Engineering Office of Admissions, Louisville, KY — Aug 2013 - Present
Recruit current high school students interested in pursuing engineering at the University of Louisville by attending college fairs around the state of Kentucky, conducting visits and information sessions on campus, and visiting local high schools

Cardinal Host, Office of the President, Louisville, KY — Jun 2011 - May 2013
Assisted at Presidential events and served as marshal for commencement ceremonies

Kentucky Spinal Cord Injury Research Center

Bioengineering Co-op, Louisville, KY — Aug 2011 - Dec 2011
Developed the hardware and software for a prototype kinetic assessment tool used to quantify overground stepping forces exerted by spinally injured rats and mice

HONORS & AWARDS

Whitaker International Program, Summer Grantee to Germany — Apr 2014
Outstanding Intern Award, NASA Johnson Space Center — Apr 2013
Citizens for Space Exploration, IN/KY Student Representative for annual trip to Washington D.C. — May 2013 & 2014
Mickey R. Wilhelm Achievement Award, University of Louisville, Department of Bioengineering — Apr 2013
Engineering Collaboration Award, Research Louisville — Oct 2011
Tau Beta Pi Engineering Honors Society — Jan 2013
Commissioned Kentucky Colonel — May 2013

ACTIVITIES

James Graham Brown Fellowship Aug 2009 - May 2013
Full tuition and expense scholarship at University of Louisville with additional assistance provided to pursue a private pilot's license program and complete a comparative health systems course in London, England & Dublin, Ireland
Biomedical Engineering Society, UofL Chapter Vice President
Global Initiatives Committee, Marketing Chair
Habitat for Humanity, Mini-Build Coordinator and Construction Volunteer

PUBLICATIONS IN PREPARATION

Spray-dried Albumin Curcumin Particles Enhances Curcumin Bioactivity.
Soucy, P.A.; Jain, I; O'Toole, M.G; Fascioto, B.H.; Hoblitzell, P.J.; Keynton, R.S.; Ehringer, W.D.; Gobin, A.S.
Gamma Irradiation Protection from Low Doses of Curcumin.
Soucy, P.A.; Fasciotto, B.H.; O'Toole M.G.; Jain, I; Keynton, R.S; Ehringer, W.D.; Gobin, A.S.

POSTER PRESENTATIONS

Controlled Curcumin Release from Spray Dried Albumin Particles Oct 2013
KY EPSCoR Annual Conference
Increasing Curcumin Solubility via Spray Dried Albumin Particles Oct 2012
Biomedical Engineering Society Annual Meeting
Curcumin Delivery from Spray Dried Albumin Particles Sept 2012
Sullivan University Annual Nanotechnology Symposium
Space Suit Assembly Engineering at NASA Johnson Space Center Sept 2012
James Graham Brown Fellows Symposium
Coupled Human-Space Suit Mobility Testing Aug 2012
NASA Johnson Space Center Innovative Research & Design Poster Session
Curcumin Uptake and Effects on Gamma Irradiated Cells (2nd Author) Oct 2011
Biomedical Engineering Society Annual Meeting
Quantifying Overground Locomotion in Spinal Cord Injured Rats and Mice Using Engineering Design Concepts Oct 2011
Research Louisville, won Engineering Collaboration Award
Curcumin Induced Death of Human Promyelocytic Leukemia Cells Apr 2011
University of Louisville Undergraduate Research Symposium